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CONTRACTOR: Marine Science Institute, University of California, Santa Barbara

CONTRACT TITLE: Host-Symbiont Interactions Between a Marine Mussel and Methanotrophic Bacterial Endosymbionts.

START DATE: 1 October 1987

RESEARCH OBJECTIVE: To delineate the interactions between a newly discovered mussel and its methanotrophic symbionts in order to reach a more complete understanding of the intact symbiosis.

ACCOMPLISHMENTS: Since the discovery and demonstration of the symbiosis between methanotrophic symbionts and a deep-sea mytilid in 1986, our efforts have been directed at determining the interactions between the symbionts and hosts which determine the properties of the association. Of particular concern has been the role of each partner in determining which external metabolites are taken up and utilized by the association and what the role of each partner is in the uptake and metabolism of these metabolites. We have been particularly concerned with the C and N sources for the symbionts and the intact association.

We have investigated the biology of this symbiosis within the context of our developing model of mussel/chemoautotroph symbioses in general as being relatively unspecialized with regard to hosts' control of the symbionts' environment. For example, unlike other groups with chemoautotrophic endosymbionts, *Bathymodiolus*-like mussels can have either sulfur-oxidizing or methanotrophic symbionts or in one recently discovered case both simultaneously. There appears to be relatively little morphological or physiological specialization associated with these symbioses in these mussels and the mussels retain the ability to utilize particulate food as well as organic material from the symbionts. However, the distributions of these mussels and the stable C isotope ratios of their tissues make it clear that the symbionts are the primary source of food for the animals under most conditions.

While it is apparent that CH_4 is the major source of carbon for the methanotrophic mussels which we studied, the nitrogen source is not so obvious. We have investigated four possible sources: Particulate organic N, dissolved organic N, NH_4^+, and direct fixation of N_2. The first two sources (particulate and dissolved), can also serve as carbon sources. These mussels' environment is rich in ammonium and free amino acids unlike the habitats of typical photoautotrophic symbioses.

The ability of these mussels to clear and assimilate organic particulates from the water column was tested for two sizes of living particles (algae~9µm and bacteria ~1µm). The results were surprising because investigators examining their anatomy and gill ciliation had communicated to us that these mussels were most likely not competent filter feeders. However, we found that these mussels can clear particles from the water and that they ingest and assimilate a significant portion of what they clear. Although not as efficient as *Mytilus edulis* (a common intertidal mussel) with algae, they are almost as efficient with smaller particles such as 1µM bacteria (Page et al., 1989).

In studies with glycine and alanine, we have found that this animal is similar to many soft bodied marine invertebrates in its ability to assimilate dissolved amino acids and similar to *Mytilus edulis* with respect to rates of amino acid uptake (Lee et al., submitted). Given the free amino acid concentrations which we have measured in the mussels' environment, FAA's may be a significant source of nitrogen for this symbiosis.

Ammonium flux kinetics were determined for both symbiotic and (functionally) aposymbiotic mussels at a range of ammonium concentrations. Symbiotic mussels in the presence of methane took up ammonium at rates ranging from 0.1 to 0.8 µmol/g/h depending on the initial concentration of ammonium. The aposymbiotic mussels excreted ammonium at rates of 0.25 µmol/g/h. From a Lineweaver-Burke plot an apparent K_s of 22.8 µM for ammonium was calculated for the intact symbiosis (Lee et al., submitted). The non-linear kinetics of this uptake support the suggestion that there may be host involvement in it. We believe that ammonium is probably the major N source for this symbiosis under normal field conditions.

Dot blots of total gill DNA for Nif structural genes gave strong positives under conditions of high stringency. This observation along with the occasionally very negative $d^{15}N$ values found in the mussels tissue ($d^{15}N$ ranges from +3 to -12.9 in animals from different sites) indicates that N_2 is also a potential source of nitrogen for the intact association, although we have not been able to demonstrate this activity in live animals or tissue preparations. This question will be addressed using $^{15}N_2$ and a new Mass Spectrometer at UCSB. The data in hand indicate that this symbiosis can indeed use a variety of N sources, although ammonium is apt to be the most important under conditions where the mussels are associated with sediments or brines. In other cases where the mussels are living on carbonate deposits, the environmental concentrations are not well enough defined yet to allow us to suggest their major N sources.

The roles of the symbiont and host properties in determining uptake kinetics for major nutrients has also been explored for methane and oxygen usage by the symbiosis. In studies of the intact symbiosis in a flow-thru GC and MS respirometer system, we have demonstrated that the uptake of these substances is very strongly affected by their concentrations with maximal uptake not occurring until concentrations near 250 µM are reached (Kochevar et al, submitted). This sort of concentration dependency is typical of all mytilid mussels and appears unchanged in these symbiotic mussels even though the metabolic demands of methanotrophy result in O_2 fluxes into the gills which are 50 fold greater than the O_2 fluxes into the gills of non-symbiotic mytilids. Thus the underlying gas exchange physiology of mytilids appears little adapted for symbiosis in the methanotrophic mussel. As a result the intact

association is strongly dependent upon environmental concentrations of CH_4 and O_2 to determine the flux rates in situ.

The stoichiometry of CH_4 oxidation observed in these experiments indicates that a substantial fraction (about 50%) of the CH_4 is assimilated into organic C in the symbiosis. This growth efficiency is in the range of the highest growth efficiencies of cultured free-living methanotrophs and indicates that the symbiosis enables the symbionts to assimilate carbon with a high efficiency. These data have also enabled us to model the environmental dependency of the symbiosis on these gases and estimate the relation between environmental levels and net C fixation by the association.

To determine the role of the symbionts in the environmental dependencies of the intact association we have used labeled methane with isolated gills and symbionts to investigate the symbionts' requirements for methane and sensitivity to oxygen. Basically we have found the symbionts do not have an abnormally low affinity for methane (the symbionts affinity is in line with free living methanotrophs and their apparent K_m is around 1 μM). Therefore, we have concluded that the significant environmental dependencies of the intact association on CH_4 and O_2 concentrations results from the limitations of mytilid physiology and not from the symbionts. These data indicate quite clearly that although free-living methanotrophs may flourish in environments with only slightly elevated methane, symbioses such as this one require substantially elevated methane concentrations (Kochevar et al., submitted).

The mode of carbon exchange in this association (translocation vs digestion) was investigated using ^{14}C-methane pulse-chase incubations. It should be noted that this association is especially well suited for investigations of this sort because methane is an unambiguous label (unlike CO_2) as it is only incorporated by the symbionts. This association has proven to be unique among autotrophic symbioses (at least among those so far investigated). Unlike results from similar studies with algal-invertebrate associations or our own results from an earlier study of the chemoautotrophic symbiosis, Solemya reidi (Fisher and Childress, 1986), these data indicate that the symbionts in these mussels do not translocate fixed carbon to their host. Over 98% of the fixed carbon is still found in the gills even after a one hour chase period. Quantitative transfer into non-symbiont containing tissues does occur after longer chase periods (one and five day), which strongly supports host digestion of the symbionts as a primary mechanism for nutrient transfer in this association.

Review of TEM's of mussel gill tissue supports this hypothesis because there are abundant lysosomes present in the bacteriocytes and numerous symbionts in various stages of engulfment and digestion in the lysosomes are visible (Fisher and Childress, submitted). Although this at first appears to be an evolutionarily primitive mode of interaction, there are some significant advantages over associations in which the symbionts only translocate bulk "junk food" to their hosts. In fact calculations indicate that it is possible that these autotrophic symbionts (using only methane and ammonium which are both abundant in their environment as C and N sources) could meet the bulk of the hosts nutritional requirements, including essential nutrients, which the host is unable to synthesize for itself. On the other hand as pointed out above, the mussels are also able to utilize both particulate and dissolved sources of organic C and N (both relatively abundant in their environment) to supplement their diet.

During an Alvin cruise to the Gulf of Mexico in early 1990, a discovery was made which further substantiates the idea of less specialized associations in the *Bathymodiolus*-like mussels than in other bivalves with chemoautotrophic symbionts. A new community of chemoautotrophic and methanotrophic symbioses were discovered at 2200 meters near Alaminos canyon in the Gulf of Mexico (Brooks et al., 1990). One species of mussel found there (closely related to the subject of this contract) has both methanotrophic and chemoautotrophic symbionts in its gills. This has been verified by enzyme assays, elemental sulfur analyses, stable carbon isotope determinations, and ultrastructural investigations. In fact both morphological types of symbionts are sometimes present in the same vacuole of the same cell (Fisher et al., in prep).

Another important substance in the environment of the methanotrophic mussels is hydrogen sulfide. This is a potent inhibitor of aerobic metabolism both in the host and in the methanotrophic symbionts. Laboratory studies of these mussels exposed to sulfide indicate that they oxidize it to thiosulfate and can tolerate up to about 100 µM concentrations. Analyses of the blood of freshly captured mussels show the presence of thiosulfate supporting that these associations have significant sulfide exposure in situ. When exposed to sulfide in the respirometer system, aposymbiotic methanotrophic mussels remain open and oxidize sulfide. This oxidation is shown by high levels of consumption of sulfide and proportional high levels of oxygen consumption. This sulfide oxidation apparently does not provide metabolic benefits to the mussels, since the CO_2 flux of the mussels is unaffected by the sulfide flux. In the near future we will evaluate the role of the mussels' sulfide oxidation in protecting the symbionts from sulfide toxicity.

In summary we now understand the ranges of environmental conditions of O_2, CH_4, sulfide, and ammonium which are necessary for the functioning of this association. We also have a good deal of information on the roles of the two partners in determining the properties of the association. Our future work on this symbiosis will be to extend this work to obtain a more complete picture of the interactions and roles of the symbionts and the hosts, especially in terms of N metabolism.

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TRAINING ACTIVITIES: Three graduate students have been funded in part by this award: R. Kochevar (male, Caucasian); E. Theusen (male, Caucasian), and R. Lee (male, Asian, English national). A recent graduate (BS, biology) has also been partially funded from this award: A. Moe (female, Caucasian).